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of Human Prostate Cancer

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13. ABSTRACT (Maximum 200 Words) In year 2, we have improved methods of reproducibly obtaining prostate tissue cores for tissue slice culture, of trimming the block face of fixed and embedded tissue slice cultures so as not to lose tissue for analysis, and have tested a number of fixation methods for optimum preservation of antigenicity. We have added additional antibodies to our repertoire to evaluate maintenance of structure and function of tissue slice cultures. Clinically relevant drugs with known activity in monolayer cell cultures and in patients have been tested on tissue slice cultures, and preliminary results suggest similar activity in tissue slices. This data supports our premise that tissue slice cultures can provide a realistic preclinical model to test experimental drugs. Currently, we are exploring immunoblot analyses of protein lysates from tissue slice cultures as a more quantitative and faster method of evaluating effects of drugs and other compounds on tissue slice cultures. Promising candidates can then be further analyzed by the more tedious technique of immunohistochemistry of fixed sections.			
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INTRODUCTION

At the present time, there is no experimental model system that accurately predicts the clinical efficacy of a drug against prostate cancer. Monolayer cell cultures, co-cultures combining stromal and epithelial cells, three-dimensional cultures in matrix supports, and xenografts are currently used to evaluate pre-clinical activity of experimental compounds. Numerous agents that kill or inhibit the growth of prostate cancer cells in one or more of these model systems have been identified, yet an effective chemotherapeutic agent for prostate cancer is still not available. A more realistic model of prostate cancer is urgently needed, and we suggest that tissue slice cultures will fill this need.

Tissue slices have been used for biochemical studies for several decades. Although typically viable for only a short period of time, tissue slices from liver, kidney, heart and lung were nevertheless used for many diverse studies, including toxicology, pharmacology, organ preservation and metabolism. In the early 1990's, methodology was developed for long term maintenance of tissue slices. However, the recent advancement that promises to extend the use of tissue slice cultures to a wide variety of organs and applications is precision cutting. This is accomplished with an automated microtome (the Krumdieck tissue slicer), which permits the rapid preparation of aseptically sliced live tissue with nearly identical dimensions. This instrument eliminates the major source of error in tissue slice work, namely, the use of slices of different and uneven thickness with irregular, non-reproducible damage at the cut surfaces. This improvement eliminates the variable of gas and nutrient exchange by diffusion that exists among unequally or unevenly cut slices.

With seed funds from a CaP CURE prostate cancer award, we purchased the Krumdieck tissue slicer and performed pilot studies to begin to master the technical challenges of prostate tissue slice culture. We used a special core borer to obtain tissue from a radical prostatectomy specimen. This core of fresh tissue was mounted on the Krumdieck microtome and thin sections were cut by a rapidly reciprocating, disposable microtome blade. Throughout the cutting procedure, the tissue remains immersed in chilled physiologic fluid. Upon cutting, the thin sections gently shoot into a reservoir of physiologic fluid, and are then transferred to grids in culture vessels with the aid of a tool that resembles a miniaturized minnow scoop. The tissue slices are rotated in a specially designed apparatus that eliminates conventional mechanical shaking, which inevitably leads to disintegration of tissue at the slice-medium interface. In our pilot study, we were able to obtain cores of fresh tissue, cut thin sections and successfully transfer the sections to culture vessels. An additional challenge was to recover the thin sections for analysis after culture. We were able to fix the thin sections, transfer them to embedding cassettes, dehydrate and embed in paraffin, and cut 5 μ m sections for histological analysis after staining with Hematoxylin and Eosin (H & E). We also performed immunohistochemical analyses to check maintenance of function.

Our proposed plan is to improve our technical abilities to prepare, process and evaluate tissue slice cultures and to develop optimal culture conditions for these tissues *in vitro*. Our long term objective is to use tissue slice cultures as a pre-clinical model system to screen therapeutic agents.

BODY

Our first designated task was to optimize preparation of prostatic tissue slices. We continue to refine this technique. In year 1 of this project, we purchased an automated tissue coring press to replace our hand-held, manual tissue coring tool. The use of the automated press enabled us to obtain uniform tissue cores of consistent and uniform diameter from surgical specimens. While the automated corer was an improvement, we still had difficulty in obtaining optimal cores from every surgical specimen. We thought that this was due to the particular constituency of prostate tissue, and to the variability and heterogeneity of constituency among specimens, presumably related to histological make-up of the specimens. For example, some tissues are quite soft while others are fibrous. One unexpected problem was that the corer became dull after only a few uses, we thought due to fibrous tissue. This necessitated precision sharpening at the manufacturer in Alabama, which caused additional expense and delays in year one. Last year, another part on the microtome required replacing. However, all equipment seems now to be in good repair and we have had no additional problems.

Another improvement has been made by boring longer tissue cores. The cores are embedded in agarose prior to placing in the tissue holder on the microtome, and the cores had been detaching from the tissue holder after only about half of the core had been sliced. We now bore longer cores from the prostate specimen and this problem has decreased. We also did a study to determine how thin we could realistically cut tissue slices. Although we could slice sections as thin as 90 μm , put them into culture, retrieve the sections after culture, fix and embed in paraffin and cut 5 μm sections for histologic analysis (Fig. 4), we find that sections cut in the range of $\sim 200 \mu\text{m}$ are easier to work with and yield more material for analysis. Therefore, we now routinely slice sections at $\sim 200 \mu\text{m}$.

Another goal of task 1 was to optimize the fixation and embedding protocol for tissue slice cultures. One problem that we have encountered is that we lose a fair amount of the tissue slice as we trim the face of the block. Our idea to prevent this was to lay the tissue slice on a piece of nitrocellulose filter paper as it is embedded in paraffin in the cassette. The filter paper could then serve as a "marker" on which to trim the block, thereby preserving the limited amount of tissue in each block. While this was good in theory, in practice it didn't work. Presumably because the filter paper is harder than the tissue, once the microtome started cutting the filter paper, the small tissue slice was pulled out of the block.

Therefore, we tested another idea, which was to paint the face of the fixed tissue slice with India ink. This helped with alignment of the block, but the ink seeps through the ducts of the tissue and somewhat obscures histologic and immunohistochemical analyses (Fig. 5). We are not completely satisfied with this method and will continue to test other ideas.

We are also still not completely happy with fixation methods and continue to work on that element of Task 1. The goal is to achieve good histological preservation without having to use Antigen Retrieval methods for immunohistochemical staining. Formalin achieves the former but then so many antibodies require the use of retrieval after formalin fixation. We had thought that the use of Histochoice fixative fairly well preserved histologic details and did not obscure antigenic epitopes, but we started having trouble getting good immunohistochemical staining. Our Histochoice fixative was quite old so we ordered some fresh, but that didn't seem to help. Therefore we conducted several studies testing a variety of fixatives. These fixatives included formalin, Histochoice, 2% paraformaldehyde, and "Hope" fixative. Cultured or uncultured tissue slices were placed in the fixatives for periods ranging from 30 minutes to 2 hours. The slices were then embedded in paraffin and sectioned at 5 μm . The tissue sections were then stained with hematoxylin and/or analyzed by immunohistochemistry.

Histological preservation was good with paraformaldehyde (Fig. 1) and formalin (Fig. 6), and adequate with Histochoice (Fig. 5). Histologic preservation with HOPE fixative, however, was horrible (Fig. 7). This fixative, made by a company in Germany, is touted to preserve antigenicity and even enzymatic activity. After we saw the list of reagents, the reason for this seemed apparent, since the HOPE solution is mainly a mixture of sugars and amino acids. An incubation in ethanol does not occur until after an overnight incubation in HOPE solution, and it seems that our tissue slices disintegrate during this time. Immunohistochemical staining with antibody against keratin 18, which usually doesn't require Antigen Retrieval, was good with all of the fixatives (Figs. 1, 5, 6 and 7). Using antibodies against keratins 5 or 14, which we know require Antigen Retrieval on formalin-fixed sections, we evaluated staining in the specimens fixed with the various fixatives (without Antigen Retrieval). Keratins 5 and 14 are present in basal cells of the prostatic epithelium. There was no staining in sections fixed in paraformaldehyde (Fig. 1) or formalin (Fig. 6). So although histologic preservation was good with both of these fixatives, these fixatives would not allow localization of keratins 5 and 14 without Antigen Retrieval. The only sections with any staining for keratin 14 were those fixed in Histochoice. The staining was spotty, however, and not completely optimal (Fig. 5). It appeared to us that staining became more complete the longer the tissue had been fixed in Histochoice (i.e., staining was better in those specimens fixed for 2 hours as opposed to 15 minutes). We now believe that perhaps Histochoice will achieve our goals if we fix the tissue slices longer, and this is now being tested.

Given the technical challenges with fixing, sectioning and staining the tissue slices, we are investigating a faster way to evaluate tissue slices. We believe that we may be able to use immunoblot analysis to follow protein expression in tissue slices with time in culture and +/- various treatments. We reason that adjacent tissue slices (which we typically cut at 150-200 μm) are fairly similar in histologic makeup. Therefore, if we cut one slice as an uncultured control, then the adjacent slice is cultured, we can make protein lysates from each and monitor cellular changes or changes in protein expression in the cultured slices. Our first step was to evaluate our ability to make protein lysates from tissue slices. We purchased a Dounce homogenizer and prepared protein lysates from tissue slices at t_0 (uncultured), and at 6 and 24 hrs after culture. Approximately 300 μg of protein were obtained from each tissue slice, and 20 μg of each sample were run on an SDS-polyacrylamide gel and transferred to a blot. Labeling with actin showed ~ equivalent levels of actin in each sample (Fig. 8), providing us with an appropriate "housekeeping" protein to compare other proteins. In year 3, we will continue to explore this technique as a rapid method to monitor the phenotype of tissue slice cultures, with confirmation by immunohistochemistry of fixed specimens of interesting results.

This past year, we also added several new antibodies to our panel of markers. We have used antibodies against cleaved caspase-3 to monitor apoptosis (see below), p63 to monitor basal epithelial cells, p53 to monitor induction of a stress response, and androgen receptor (AR) to monitor differentiation. We have also ordered an antibody to monitor hypoxia in the tissue slices in future studies.

The second task was to develop optimal culture conditions for the maintenance of tissue structure and function. In the first year, we determined that "Complete PFMR-4A", consisting of defined basal media supplemented with cholera toxin, epidermal growth factor, insulin, phosphoethanolamine, hydrocortisone, selenium, alpha-tocopherol, retinoic acid, bovine pituitary extract, and gentamicin, maintained fairly good histological and functional preservation

of tissue slices cultured up to 24 hours. Basal epithelial cells, secretory epithelial cells and stromal smooth muscle remained intact, as demonstrated by immunohistochemical staining with appropriate markers. We found that the degeneration of secretory cells that began to appear by 48 hours could be lessened if androgen (10 nM R1881) was included, so we now routinely use Complete PFMR-4A with 10 nM R1881. These experiments addressed **Task 3, which was to test the validity of tissue slice cultures as an accurate in vitro model by depriving the tissue slices of androgen.** While our initial studies suggest that secretory cells are maintained longer in the presence of androgen, we want to follow-up these studies in the coming year with additional immunohistochemical studies.

We have started pilot studies to address **Task 4, to test agents used clinically for their effects on tissue slice cultures.** Docetaxel is one of the leading chemotherapeutic agents used to treat prostate cancer, so we treated tissue slice cultures +/- 1 μ M docetaxel for 48 hours. Histologic evaluation suggested an increased level of apoptosis in tissue sections treated with docetaxel compared to untreated tissue slices (Fig. 2). This was confirmed by immunohistochemical labeling with antibody against cleaved caspase-3, a marker of apoptosis (Fig. 3). We also tested doxazosin, an alpha-adrenoceptor antagonist that is used to treat benign prostatic hyperplasia. In monolayer cultures, doxazosin induces apoptosis in prostatic epithelial cells. Histological observations of tissue slices treated for 48 hr +/- 50 μ M doxazosin suggested to us that there was more cellular degeneration in the treated than in the untreated tissue culture slices (Fig. 2). We will follow this up in future studies with markers of apoptosis, such as cleaved caspase-3.

KEY RESEARCH ACCOMPLISHMENTS

- Improved method of reproducibly obtaining tissue cores from prostatectomy specimens (obtaining longer cores of tissues so that they do not detach from the tissue holder while cutting in the microtome)
- Inked the face of the tissue slices prior to embedding in paraffin to avoid loss of tissue when trimming the block
- Continued testing optimal methods of tissue slice fixation to preserve antigenicity – Histochoice seems to be best option but requires further testing
- Purchased additional antibodies to evaluate maintenance of structure and function in tissue slice cultures (androgen receptor, p53, p63, hypoxia, cleaved caspase-3)
- Evaluated the effects of clinically relevant drugs, doxazosin and docetaxel, on tissue slice cultures – observed an increase of apoptosis in docetaxel-treated tissues by using the apoptosis marker, cleaved caspase-3
- Began testing immunoblot analysis of protein lysates of tissue slice cultures as a quicker way of evaluating effects of drugs and other factors

REPORTABLE OUTCOMES

None.

CONCLUSIONS

While this technology continues to be challenging, we have overcome a number of problems. We can reproducibly obtain tissue cores of uniform size which remain attached to the tissue holder during slicing, we can cut sections as thin as 90 μm which can be cultured, recovered and analyzed, and we can maintain tissue structure and function fairly well for up to 48 hours of culture. We have tested a number of fixation methods and have chosen Histochoice as the most promising for preserving histology fairly well while also still preserving antigenicity, but we want to further investigate optimal fixation times. We have added several antibodies to our repertoire and now we routinely use keratin 14, keratin 18, PSA, AR, smooth muscle actin and cleaved caspase-3 to monitor preservation of basal and luminal epithelial cell structure and function, smooth muscle, and apoptosis. Using doxazosin and docetaxel as clinically relevant drugs whose activity is known in monolayer cultures of prostatic epithelial and stromal cells and in patients, we tested these drugs for their effects on tissue slice cultures. Both drugs appeared to cause increased apoptosis in the epithelia of the tissue slice cultures, as might be anticipated from prior information of cell cultures and clinical activity. This finding supports our contention that tissue slice cultures can provide an alternate and perhaps more realistic preclinical model system, and in the coming year, we will test additional clinically relevant agents. We are testing immunoblot analysis of protein lysates of tissue slice cultures as a faster way of screening the slices for alterations in phenotype in response to drugs or other factors. This methodology is less cumbersome than immunohistochemical analysis of these sections of fixed and embedded tissue slices, and we can use this more tedious technique to follow up interesting results from immunoblot analyses.

REFERENCES

None.

APPENDICES

Figure legend and Figures 1-8.

FIGURE LEGENDS

Figure 1. Tissue slices were sliced at ~ 200 μm and then fixed in paraformaldehyde for 1 or 3 hr. Following embedding in paraffin, 5 μm sections were cut and immunohistochemical staining for keratins 5 and 18 was performed. Sections were counterstained with H&E. The dark brown staining reflects binding of antibody (the brown staining in the top photos is background staining in the sections not exposed to primary antibody). While K18 was visualized in the secretory cells of the paraformaldehyde-fixed sections, K5 was not seen in the basal cells, where it should appear. This lack of K5 reflects antigen masking by the fixative.

Figure 2. Tissue slices were cultured for 48 hr in medium +/- 50 μM doxazosin or +/- 1 μM docetaxel. Evaluation of H&E-stained sections following fixation and embedding in paraffin suggested greater epithelial degradation in the drug-treated slices than in the control tissues.

Figure 3. Immunohistochemical evaluation of cleaved caspase-3, a marker of apoptosis, was performed to confirm greater apoptosis in docetaxel-treated tissue slices that was suggested in the H&E-stained tissues (Fig. 2). No positive staining was seen in the untreated tissue slices, whereas areas with numerous caspase-3-positive cells were seen in the docetaxel-treated tissue slices. Docetaxel increases apoptosis in monolayer cultures of prostatic epithelial cells and in patients, so this result confirms the potential utility of tissue slices as a preclinical model to evaluate drugs.

Figure 4. In order to determine how thin tissues could be sliced and be successfully cultured and retrieved for analysis, slices were made at from 90 to 300 μm . Even the 90 μm slices held up in culture and through the various stages of fixation, embedding in paraffin, and sectioning at 5 μm .

Figure 5. Tissue slices were cut and directly fixed in Histochoice for 15 to 120 minutes. Immunohistochemical staining for PSA and K18 was very good, whereas K14 (basal epithelia, shown by arrows) was spotty. K14 requires Antigen Retrieval in formalin-fixed specimens, so visualization of K14 at all without retrieval was good. Since K14 staining seemed less spotty in the tissues fixed for 120 minutes compared to 15 minutes, perhaps even longer fixation in Histochoice will allow full visualization of K14 staining.

Figure 6. Tissue slices were cut and fixed in formalin for 15 to 120 minutes. K18 is readily visualized in formalin-fixed sections, but K14 is not because of epitope mapping. We had hoped that the briefer 15 minute fixation might preserve K14 antigenicity, but this was not the case.

Figure 7. Tissue slices were cut and fixed in HOPE fixative, which is purported to preserve antigenicity. This may be the case, and certainly staining for K18 and PSA was good, but histological preservation was awful and the cells degenerated during the fixation.

Figure 8. Immunoblot analysis of protein lysates prepared from uncultured tissue section (t0) or adjacent sections cultured for 6 or 24 hours. Equal amounts (20 μ g) of each lysate were separated by SDS-polyacrylamide gel electrophoresis and transferred to a blot, which was hybridized to an antibody against actin. The equivalent levels of actin in the samples show that we can use actin as a protein to which we can compare levels of other proteins that are likely to be modulated by culture and/or drug treatment.

Fig. 1



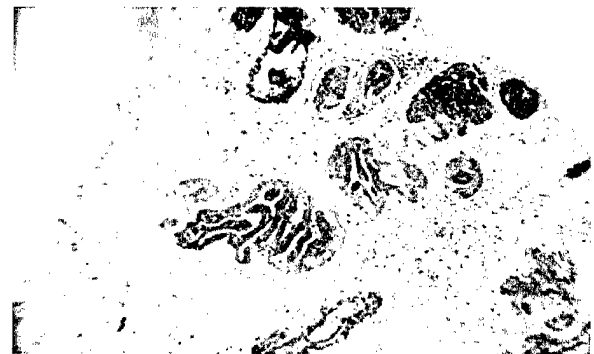
paraformaldehyde 1 hr



paraformaldehyde 3 hr



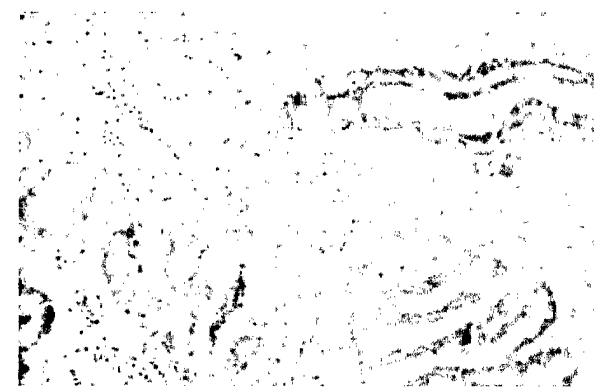
paraformaldehyde 1 hr K18



paraformaldehyde 3 hr K18

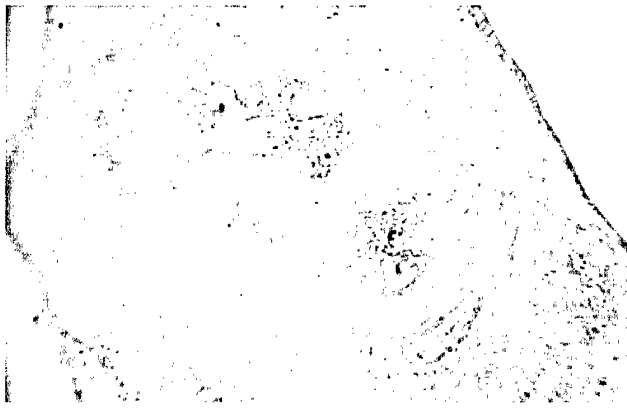


paraformaldehyde 1 hr K5

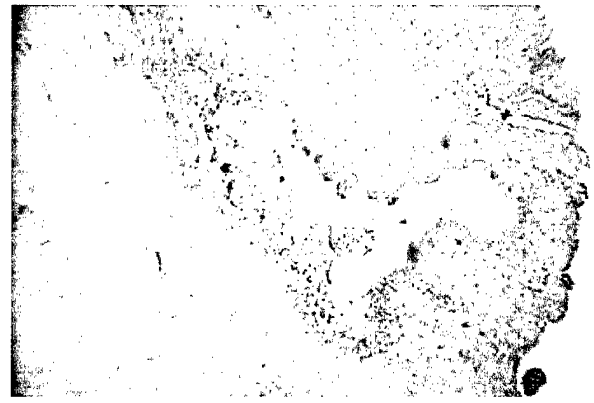


paraformaldehyde 3 hr K5

Fig. 2



0 doxazosin



50 μ M doxazosin

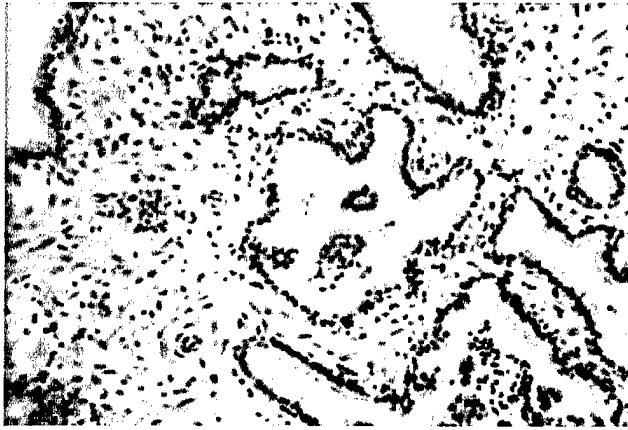


0 docetaxel

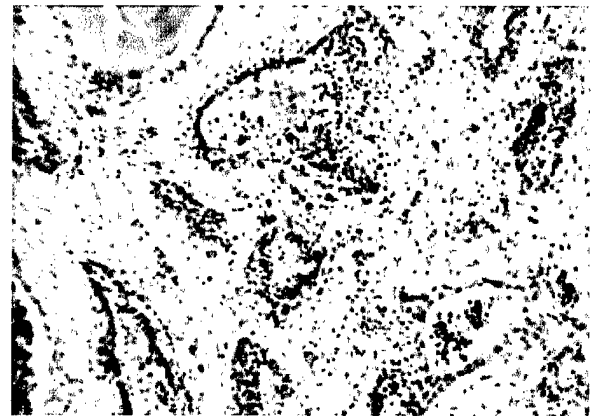


1 μ M docetaxel

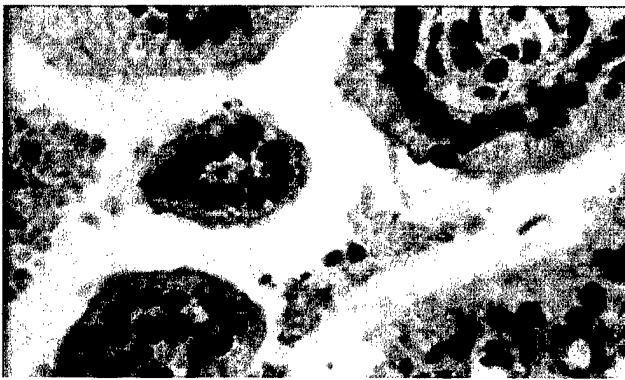
Fig. 3



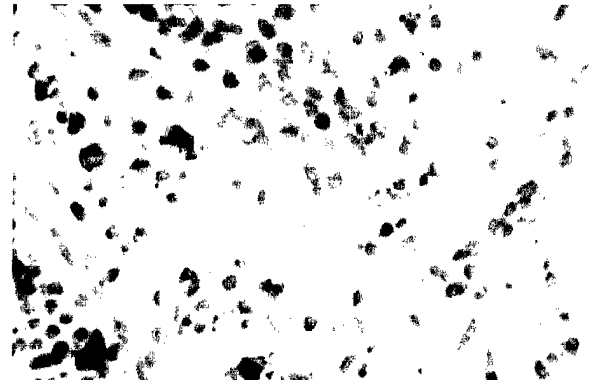
0 docetaxel cleaved caspase



1 μ M docetaxel cleaved caspase

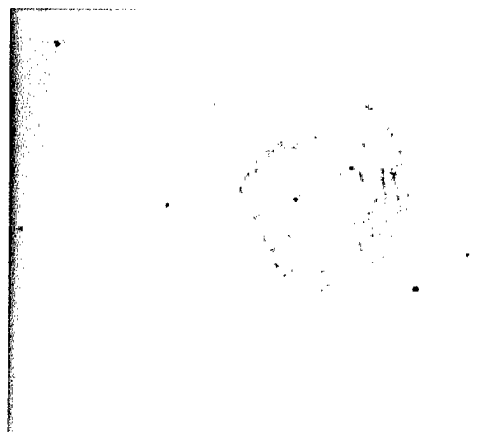


0 docetaxel cleaved caspase (40X)



1 μ M docetaxel cleaved caspase (40X)

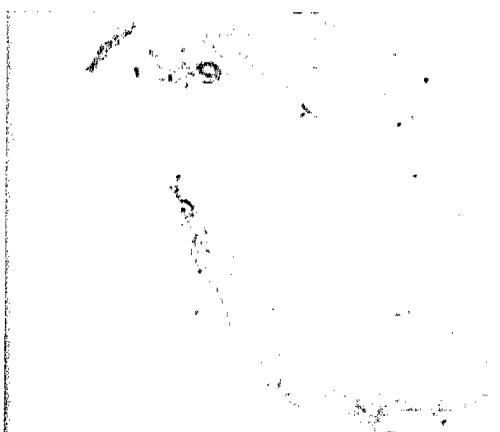
Fig. 4



300 μ m



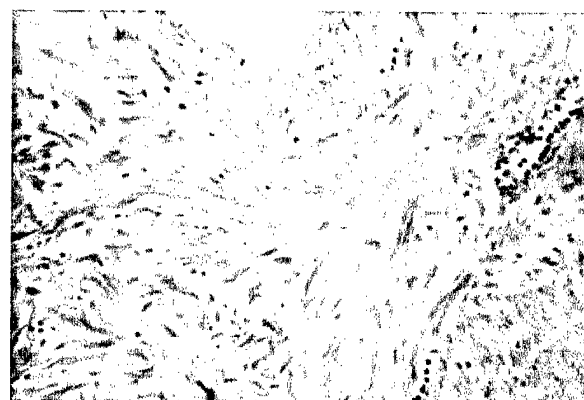
250 μ m



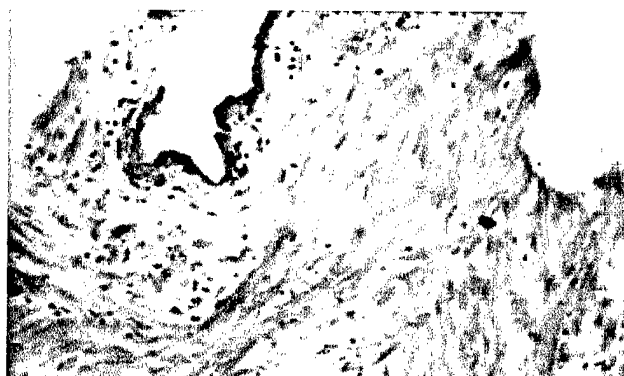
150 μ m



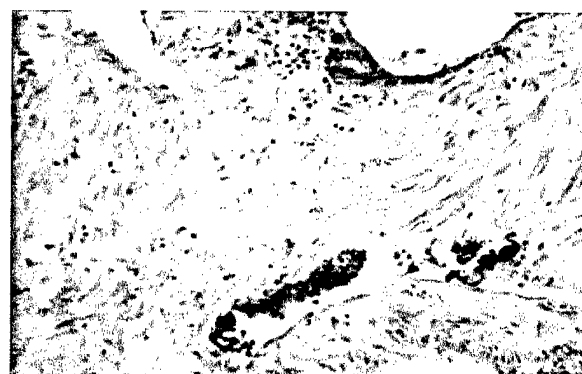
100 μ m



100 μ m culture



90 μ m



90 μ m culture

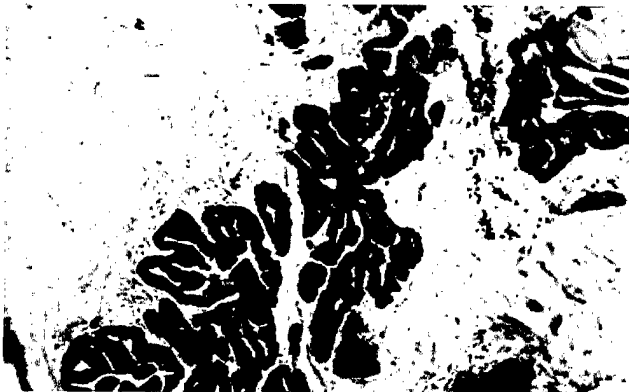
Fig. 5



Histochoice 15' PSA



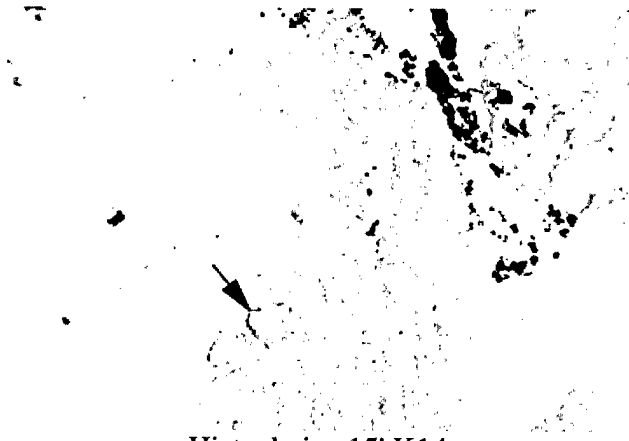
Histochoice 120' PSA



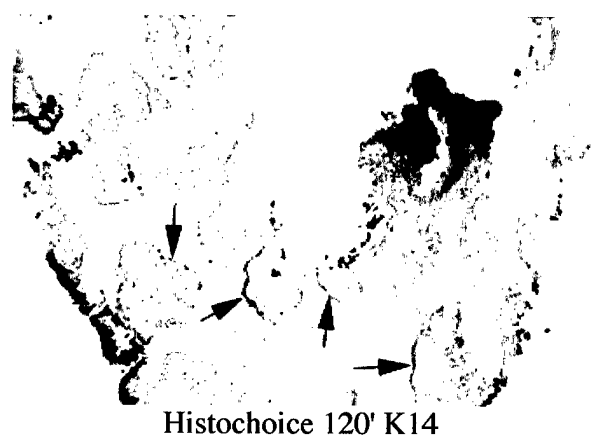
Histochoice 15' K18



Histochoice 120' K18

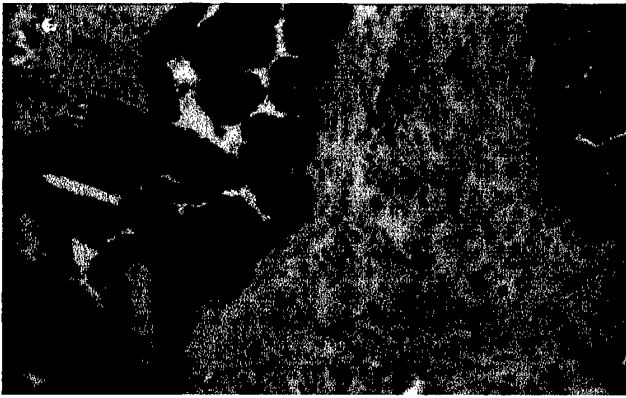


Histochoice 15' K14

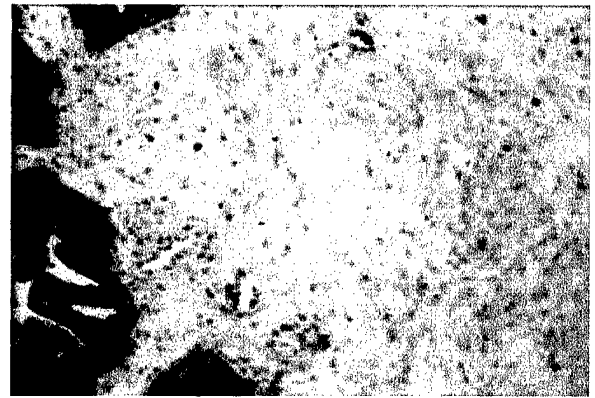


Histochoice 120' K14

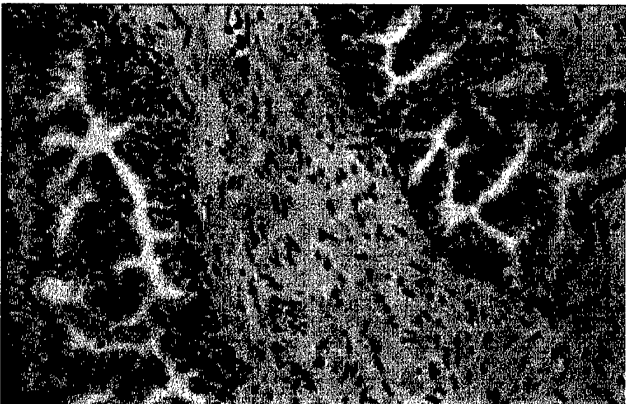
Fig. 6



formalin 15' K18



formalin 120' K18

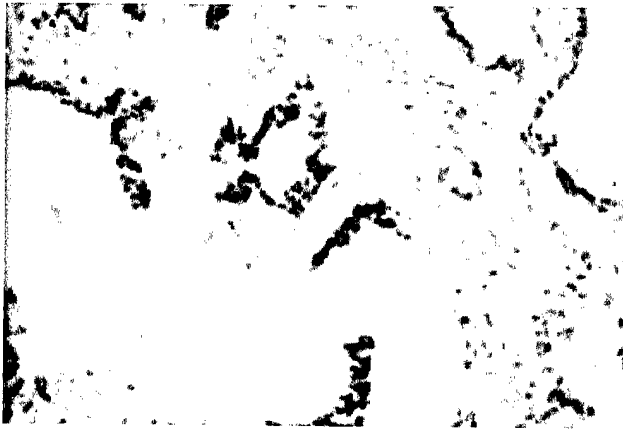


formalin 15' K14

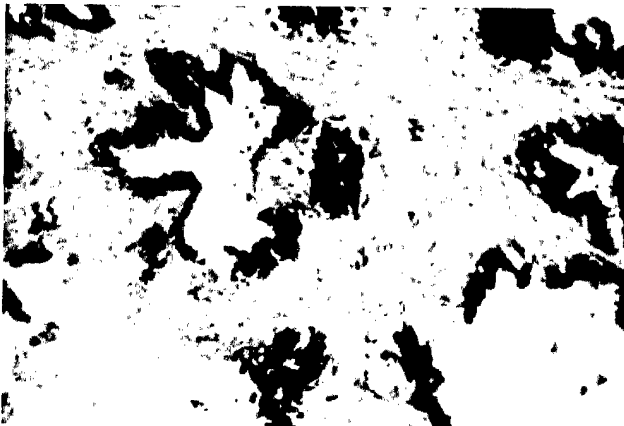


formalin 120' K14

Fig. 7



HOPE

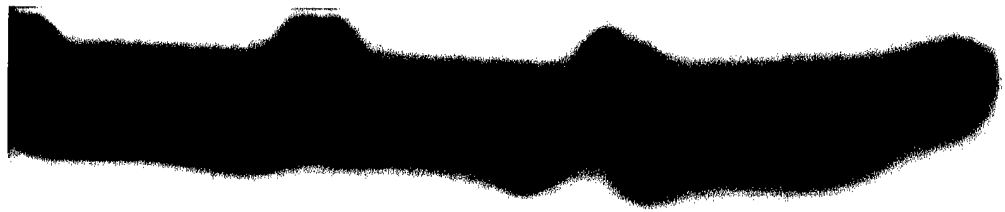


HOPE K18



HOPE PSA

Fig. 8



0 hr

6 hr

24 hr